#972

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1-31-74

1. Principal Investigator (give title and degrees):

Marvin A. Friedman, Assistant Professor of Pharmacology
S.B., S.M., Ph.D.

2. Institution & address:

Department of Pharmacology
Medical College of Virginia
Health Science Division
Virginia Commonwealth University
Richmond, Virginia 23298

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Suppression of Dimethylnitrosamine and 3-Methylcholanthrene Carcinogenicity by Nitrogen Dioxide

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: June 30, 1977

7. Brief description of specific research aims:

1. Investigate the correlation between atmospheric NO_2 levels and methemoglobin levels in mice.

2. Quantitate hepatic and pulmonary mixed function oxidase activity subsequent to NO_2 exposure.

3. Determine the kinetics of the effects of NO_2 on mixed function oxidase activity and the effects of NO_2 on cytochrome P-450.

4. Determine the effects of NO_2 on liver, lung, and renal DMN demethylase activity and liver, intestine, lung, and skin aryl-hydrocarbon hydroxylase activity.

5. Investigate the effects of NO_2 on mutagenicity of DMN on the host mediated assay and 3-MC in the dominant lethal test.

6. Determine the effects of NO_2 on transformation of lung cells in vitro by 3-MC or transformation of hamster embryo cells by 3-MC in vitro and by 3-MC and DMN in the host-mediated carcinogenesis assay.

7. Determine the effects of NO_2 on lung and systemic carcinogenic responses to 3-MC and DMN.

8. Determine the effects of NO_2 on 3-MC applied by skin painting.

8. Brief statement of working hypothesis:

Since NO_2 induces methemoglobinemia in experimental animals, we feel that it will have some biochemical properties in common with sodium nitrite, a compound which induces methemoglobinemia in an identical fashion. The particular biochemical lesion in question is the inhibition of liver mixed-function oxidase activity. Our working hypothesis is, therefore, that NO_2 will inhibit mixed function oxidase activity. The direct corollary of this is that NO_2 will also suppress DMN demethylase and aryl-hydrocarbon hydroxylase activities both of which are mixed-function oxidases and both of which activate carcinogens to their proximate form. The end result of this will be protection from mutagenic and carcinogenic effects of dimethylnitrosamine and 3-methycholanthrene. We, therefore, feel that this component of side-strain cigarette smoke will protect people from the carcinogenic effects of food contaminants and smoke components.

9. Details of experimental design and procedures (append extra pages as necessary)

It is the purpose of this research to evaluate the effects of long term exposure to NO_2 other than the pulmonary toxicity which results from direct action of nitric acid on the lung. The basis for anticipating a systemic response is that NO_2 induces methemoglobinemia. We have shown that sodium nitrite, which induces methemoglobinemia in an identical fashion as NO_2, suppresses mixed function oxidase activity. Although this induction of methemoglobinemia by NO_2 is well established, there are no published dose-response data for this phenomenon. Therefore, it is important to establish dose-response relationships between atmospheric NO_2 concentrations and methemoglobin levels. With this relationship established, we will be able to better interpret experimental data, and we could better establish whether subclinical methemoglobinemia is a public health concern. Subacute and chronic levels of methemoglobin may then be related in a quantitative fashion to atmospheric NO_2 levels. These dose-response relationships will also be important for setting up protocols for later experiments.

Once methemoglobin levels are established, we will test for methemoglobin mediated suppression of hepatic mixed function oxidase activity. NO_2 would be expected to inhibit liver enzyme activity in a fashion identical to sodium nitrite. Typical Type I and Type II enzymes-namely, aminopyrine demethylase and aniline hydroxylase, will be studied. Enzyme activity will be determined in lung as well as liver because lung is the important site for neoplastic transformation. Since there is a direct effect of NO_2 on lung tissue, one would expect the lung enzymes to be more accessible as well as more severely effected.

In order to study the chronic effects of NO_2, maximally tolerated levels must be established in a two month subacute experiment. Groups of animals will be exposed to NO_2 for two months and gross pathology taken to determine what doses will be used in later experiments. Once the levels of exposure are determined, the carcinogenicity studies will begin. DMN will be fed to mice who are exposed to varying levels of NO_2. DMN will be used in these experiments because it induces lung tumors. In addition, DMN induces liver tumors. Therefore, both the local effects of NO_2 on DMN oncogenesis in lung and the systemic effect of NO_2 on DMN oncogenesis in liver can be studied. In addition, DMN is synthesized in vivo from sodium nitrite and secondary or tertiary amines. It is thus apparent that the same populations of people living in industrialized cities who are exposed to high levels of NO_2 also consume preserved and stored foods which contain sodium nitrite or DMN.

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9. Continued

In order to support conclusions drawn from interpretation of DMN data, the oncogenic response to another common pollutant must be studied. We chose 3-MC for many of the same reasons we selected DMN. It induces systemic tumors as well as lung tumors. There is a relationship between levels of enzymes which metabolize it and neoplastic response. It is commonly occurring although more often associated with SO_2. However, automobile exhaust, the source of most urban NO_2 is also the source of a considerable amount of 3-MC. In addition, 3-MC is a human carcinogen so these observations are relevant to human exposure. 3-MC has topical activity as well as systemic activity. We can, therefore, determine whether NO_2 modifies epidermal responsiveness to polycyclic hydrocarbons.

There are apparent problems in interpretation of the in vivo reactions. The susceptibility of the animal to infection will be increased, appetite modified, behavior changes, and so forth. Therefore, it is important to support our conclusions by in vitro observations. We will expose lungs in vitro to polycyclic hydrocarbons either alone or in combination with varying levels of NO_2. This system correlates very well with in vivo responses in the sense that agents which are active in the lungs in vitro are also active in vivo and lungs from strains of mice which are resistant to lung tumors are also resistant in vitro.

We will also test for interactive responses between NO_2 and polycyclic hydrocarbons in trypsinized hamster embryo cells in primary or secondary culture. Although this system is not as conceptually related to the in vivo system, there are many similar metabolic considerations, and it is predictive in predicting carcinogenic potential of polycyclic hydrocarbons. We will bridge the gap between these studies and the in vivo studies by determining the effects of NO_2 on DMN and 3-MC transformation in the host-mediated carcinogenesis test.

It is noteworthy that we have not indicated that we will test DMN in vitro. There is no background literature indicating that DMN is active in in vitro carcinogenicity tests. We will of course test DMN, both alone and in combination, but the basis for instigating these tests is not as sound as in the case of polycyclic hydrocarbons. However, we will test DMN in the host-mediated carcinogenicity test for which data indicates that DMN is positive. Due to the rapidity of the responses, the low cost of the experiments and ease of performing these experiments, other carcinogens may be tested in vitro or in the host-mediated carcinogenicity test for synergy or antagonism with NO_2. In addition, other gaseous air pollutants such as SO_2, SO_3, or CO may also be tested. These are inexpensive experiments which will provide a great amount of information.

It has been our opinion that mutagenic potential of environmental pollutants may represent as great a potential public health hazard as the carcinogenic potential. Therefore, we will also evaluate the interactive effects of NO_2 on DMN and 3-MC mutagenicity. One of the problems in these mutagenicity experiments as that DMN is a potent mutagen in the host-mediated assay but inactive in the dominant lethal test while 3-MC is inactive in the host-mediated assay but active in the dominant lethal test. We will therfore, set up and perform the relevant test for each compound. There are other advantages to these tests. In the case of the host-mediated assay, we can determine whether NO_2 will reach in body fluids to produce mutagenic nitrosamines. As we indicated

earlier, this is a sensitive system, detecting inhibition of DMN mutagenicity at doses as low as 16.5 mg/kg sodium nitrite. In the case of the dominant lethal test, we will be able to determine if NO_2 has reproductive toxicity. We will routinely measure fertility index as part of our dominant lethal test. If it appears that NO_2 has reproductive toxicity, we will then perform a three generation reproduction test to quantitate the magnitude of the response.

Finally, we must establish directly the mechanism of the responses to NO_2. We will measure P-450 levels and spectra to determine how NO_2 is modifying mixed function oxidase activity. We will look not only for P-450 levels but also for characteristic nitroso groups which will indicate that NO_2 nitrosates P-450. We will also determine the direct effects of NO_2 on the enzyme which metabolize these carcinogens. In the case of DMN, DMN demethylase will be assayed from liver, lung, and kidney because these are the sites of neoplastic transformation. This enzyme will be measured in vitro by assay of formaldehyde produced from incubation of liver microsomes with DMN in the presence of relevant cofactors or alkylation of protein and nucleic acids by DMN-C^{14}. Analogous experiments will be performed to determine the effects of NO_2 on aryl-hydrocarbon hydroxylase. In vitro studies to determine the aryl-hydrocarbon hydroxylase activity in the liver, intestine, lung, mammary gland, and skin will be determined. Intestine, lung, mammary gland, and skin will be studied because these are sites of neoplastic transformation. Liver will be studied because it is the site of catabolism of polycyclic hydrocarbons prior to excretion. For example, inhibition of liver enzyme activity will increase the 3-MC half life in the animal and increase the possibility of neoplastic transformation. Increases in liver enzyme activity by DDT have been shown to decrease the oncogenicity of 3-MC.

The strain of mouse to be studied is very important to interpretation of the experiments. We have a broad spectrum to choose from ranging from C57BL which is markedly resistant to lung tumors to the Strain A mouse which is sensitive to lung tumors to the AKR which carries a leukemia virus activated by 3-MC. The two strains of mice we have most experience with are ideal for these studies because they are sensitive to the lung carcinogenicity of 3-MC and DMN and are hearty, strong animals which can tolerate the air pollutants. We routinely use Swiss mice because of cost consideration, health considerations, (they are an infection-resistant mouse) and extrapolation of data to a ramdom bred human population. They are random bred mice so we feel they are most characteristic of the population responses, and the data is more relevant for humans. This strain will be the mouse used in the bulk of the studies. We often need an inbred mouse for problems of tumor biology, and we have selected the C3H/HE. This is a good inbred strain which is sensitive to lung carcinogenicity by 3-MC. This strain does not carry the Bittner factor for spontaneous mammary tumors. These animals will be used for in vitro tests and to confirm the results obtained in studies on Swiss mice. There will also be occasion to study a resistant strain and in these studies C57BL mice will be used.

It will also be essential to have accurate data on urban levels of NO_2 so that the dose response data obtained here can be interpreted in view of community health. This data will be obtained by the Mobile Unit at the Richmond Air Pollution Control Center. With experimental dose-response data and accurate measurements of the levels of pollutants in urban areas-namely, Richmond-we can evaluate the significance of ambient NO_2 levels.

METHODS OF PROCEDURE

1. Animals

Male Swiss (ICR) mice will be used unless otherwise stated and are available by direct truck from Flow Laboratories in Dublin, Virginia. C3H/HE mice will be used in some experiments and also are available from Flow Laboratories. C57BL mice when required will be obtained from Sprague Dawley in Madison, Wisconsin. Mice will be housed in shoebox-type plastic cages covered by disposable filters. Mice will be maintained on agar gel diets containing the carcinogens (1). We have found agar gel diets are a very safe and efficient way to handle toxic diets. There is a sufficient variation in stock diets that a semipurified diet is almost essential for interpretation of long-term experiments.

There will be two types of inhalation chambers used in these studies. The geometrics of the exposure system is not a matter of great concern since NO_2 is a freely diffusible gas and does not settle and cannot be filtered through fur. For acute studies, systems resembling the cylindrical glass battery as described by Leach and for chronic studies, chambers similar to the New York University model will be used (2). The cylindrical-type cages, 4" in diameter and 12" long, can be readily constructed at the Medical College of Virginia and will be ready at the point of initiation of these studies. For chronic experiments, the design is more complex. A plexiglass box, 3' by 3', opening on one side and with three shelves will be constructed here at M.C.V. The bottom and top will be constructed as pyramids to insure an even air flow. The smaller cages can be used for the subacute experiments, and no time will be lost in construction of these cages. We have at the Medical College of Virginia necessary plastic cages and accessories for the rest of the study.

2. Environmental NO_2 levels:

The mobile lab at the Richmond Air Pollution Control Center is currently set up to measure ambient NO_2 levels by established E.P.A. procedures (3). In collaboration with this project, the mobile unit will be stationed at high traffic areas in the City of Richmond to measure NO_2 levels. Additionally, levels of exposure of experimental animals will also be constantly monitored by procedures we find are better suited for routine laboratory analyses (4).

3. Microsomal Mixed Function Oxidase Determinations and Methemoglobin Assays

Methemoglobin levels will be determined on periferal blood isolated from the tail. The percentage of methemoglobin will be determined by the difference in absorbancy of blood before and after addition of sodium cyanide (5). Both the time course and the dose response to NO_2 will be determined.

Mouse liver, kidney, and lung mixed function oxidase activities will be determined following chronic and acute exposure to NO_2. Aniline hydroxylase and aminopyrine demethylase activities will be determined.

Assays for drug metabolizing enzyme activity will be performed on crude microsomes. Mice will be killed by decapitation, and the test organs will be removed and chilled. Following homogenization in sucrose buffer, samples will be centrifuged at 10,000 x g for 15 minutes. Preparations isolated in this manner are then stable to freezing, if necessary. Determinations of the activity of several different enzymes can be performed on the same microsomal enzyme preparation.

The standard incubation mixture we have employed in previous experiments with sodium nitrite contained 75 u moles nicotinamide, 4.5 u moles NADP, 15 u moles $MgCl_2$, 30 u moles $MnCl_2$, 15 u moles D, L isocitrate, and 75 gm isocitric dehydrogenase and 0.3 ml of microsomal preparation. Following incubation at 37° in a Dubnoff shaker, the reactions are stopped and enzyme activity quantitated. Microsomal aniline hydroxylase activity is quantitated by measuring p-aminophenol production while aminopyrine demethylase is assayed by determining formaldehyde production (6).

The dose-response of liver and lung mixed function oxidase activities to single 45 minute exposure to varying levels of NO_2 from 50 ppm to 0.4 ppm will be determined. Similarly, the time course of onset of effects from a single 45 minute exposure to 50 ppm NO_2 as well as the duration of effects from the exposure will also be determined. Subacute studies will then be performed to test whether these effects are cumulative.

In subsequent experiments the effects of NO_2 on Km and Vm of lung and liver mixed function oxidases will be determined at times of maximum suppression of enzyme activity (6). These data will indicate whether the inhibitory effects of NO_2 are competitive, uncompetitive, or noncompetitive. Then cytochrome P-450 levels and spectra will be determined (7) in order to establish whether the observed effects are mediated through cytochrome P-450.

4. Aryl-Hydrocarbon Hydroxylase and DMN Demethylase Determinations

The acute, subacute, and chronic effects of NO_2 exposure on aryl-hyrdocarbon hydroxylase and DMN demethylase activities will be determined. Aryl-hydrocarbon hydroxylase will be measured by determining conversion of benzpyrene to 3-hydroxybenzpyrene (8). In some studies 3-MC-6-C^{14} will be added and its metabolic products identified. DMN demethylase will be assayed by quantitating the methylation of protein and nucleic acids following incubation with DMN-C^{14} (9). We will also perform formaldehyde determinations to measure the enzyme activity. In all cases the incubation medium and enzyme sources described for aminopyrine demethylase and aniline hydroxylase will be used for these studies.

Acute, subacute, and chronic studies of the effects of NO_2 on aryl-hydrocarbon hydroxylase and DMN demethylase will be performed. Enzyme assays will be performed at periods when aminopyrine demethylase and aniline hydroxylase are suppressed. Enzyme assays will be performed following single exposure to NO_2 and at monthly intervals in experiments under conditions identical to those in the long term studies. In the case of aryl-hydrocarbon hydroxylase, enzyme activity in intestine, liver, lung, and skin will be measured (10). In the case of DMN demethylase, liver, kidney, and lung enzyme activity will be measured.

5. Mutagenicity Testing

We will test for reverse mutation of S. typhimurium G-46 in a host mediated assay (11) This system has shown that sodium nitrite antagonizes DMN mutagenicity (12). Groups of 10 mice will be injected I.P. with S. typhimurium G-46 and sacrificed three hours later. The organisms will be recovered, diluted appropriately, and plated. All animals will be run individually.

Mice will be exposed to NO_2 in either acute dose-response or chronic experiments. Then each animal will be treated I.M. with DMN and I.P. with bacteria. Relevant solvent and unexposed mouse controls will be performed. Mutant frequence in recovered S. typhimurium will be determined.

We will also determine the effects of NO_2 in the dominant lethal test (13). Groups of 10 male mice will be exposed either acutely or chronically to NO_2 and mated with two virgin females per male each week for eight weeks. Females will be sacrificed 14 days after initiation of mating and number of pregnancies, inplantation sites, and early fetal deaths recorded. Groups of mice will be treated with 3-MC either alone or in combination with NO_2.

6. In Vitro Carcinogenicity Tests

Interactions between NO_2 and 3-MC or DMN will be tested in vitro in two test systems. In one system (14), 12-14 day old fetuses of randombred Syrian hamsters will be grown in grown Eagle's minimum essential medium with 10% calf serum and exposed to 3-MC (5 ug/ml) in the dark. Cells will be plated in complete medium with 10% bovine serum in a petri dish containing 6×10^4 irradiated rat embryo cells. Hamster cells will be seeded on cover slips adjusted to cover most of the surface of the petri dish. Nine days after addition of 3-MC, the dishes will be examined with phase microscopy for transformed colonies. Some plates will be fixed and stained with Giemsa for further analysis. All incubations will take place under controlled relative humidity, 10% CO_2 37°C and varying concentrations of NO_2. The pH will be monitored at all times since NO_2 is acidic.

The synergistic or antagonist carcinogenic potential of NO_2 will also be evaluated in the host-mediated in vivo - in vitro assay for chemical carcinogenesis (15). Pregnant Syrian golden hamsters at 10 to 11 days gestation will be injected I.P. with 5-30 mg/kg DMN or 100-300 mg/kg 3-MC. These animals will be exposed to NO_2 either acutely in time-response experiments or in dose-response experiments or chronically for 1-6 months prior to mating. The hamsters will be killed 48-72 hours after treatment (on Day 13 of gestation), and the fetal cells cultured in Eagle's medium with 10% fetal bovine serum. These cells will be passed in a ratio of 1:10 every 4-6 days for at least three passages.

In a second series of experiments, lungs from one month C3H/He mice will be exposed in vitro to 3-MC and implanted into mice of the same strain (16). Whole lungs from one month old mice will be cut into pieces approximately 2 x 1 x 1 mm and cultured on strips of cellulose acetate in petri dishes containing trowells T8 culture media with 15% added C3H serum. Solutions of 3-MC in acetone will be added to the medium to give final concentrations of approximately 5 ug/ml. Explants will be cultured for one day in control medium, four days

on carcinogen containing medium, and one day in control medium. These explants will then be implanted subcutaneously in six week old C3H/He mice and the animals observed for up to 12 months. The variables tested in these experiments involve: 1) The effects of NO_2 in vitro on 3-MC transformation of lung cells, and 2) The sensitivity to in vitro transformation of lung cells from animals previously exposed to NO_2.

7. Lifetime Carcinogenicity Studies

The following groups and numbers of mice will be exposed for their entire lifetime to NO_2 either alone or in combination with a carcinogen.

Group	NO_2 Exposure	Carcinogen Exposure
I	none	none
II	high dose ca 3 ppm	none
III	low dose ca 0.5 ppm	none
IV	none	DMN in diet
V	high dose	DMN in diet
VI	low dose	DMN in diet
VII	none	3-MC in diet
VIII	high dose	3-MC in diet
IX	low dose	3-MC in diet
X	none	3-MC topically
XI	high dose	3-MC topically
XII	low dose	3-MC topically

In the case of Groups X, XI, and XII where induction of skin tumors is one of the end points, some experimental conclusions may come as early as six months allowing dose-response experiments to be performed as repeats. Doses of 3-MC will be applied in acetone solution to the backs of shaved mice. Mice will then be shaved at weekly intervals and inspected for papillomas.

Doses of NO_2 will be determined in two month preliminary dose response experiments. The high dose used will be the maximally tolerated dose(i.e. the dose below which no weight loss occurs). The low dose will be 1/10 the maximally tolerated dose. DMN will be fed in agar diets at a level of 5 ppm which gives a spectrum of tumors in mice (17), and 3-MC will be fed at 10 ppm which also gives a broad spectrum of tumors in mice (18). Mice will be inspected twice daily weekdays and on weekends for tumors or morbidity or mortality. Dead or morbid animals will be autopsied and liver, spleen, lung, and G.I. tract taken for histopathology. Body weights will be recorded weekly for the first six weeks and then monthly starting at eight weeks. Mice will be exposed to NO_2 for eight hours daily, seven days per week. The limited exposure is to insure that the food and carcinogens in the food are not effected by NO_2.

8. Potential Interactions Among Other Gases

In the latter stages of the project, when we have exposure chambers standardized, and most of the data on NO_2, we will test other gases for acute effects. We will study CO and SO_2 primarily either alone or in combination with each other or NO_2. We will also investigate NO either alone or in combination with NO_2. We will test these gases for effects on liver and lung

DMN demethylase and aryl-hydrocarbon hydroxylase and on transformation of fetal hamster cells by DMN or 3-MC in the host-mediated carcinogenesis system. It is important to note that in these experiments as well as all previous experiments, non-carcinogen treated controls will be performed so that we will have an accurate index of the biochemical effects of these gases alone or in combination with each other.

References

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2. Described by Drew, R.T., and Lasken, S., in Methods in Animal Experimentation.
3. Mulnik, J., Fuerst, R., Guyer, M., Muker, J., and Sawicki, E., To be published.
4. This will be done in collaboration with the Richmond Air Pollution Control Center.
5. Stolman, A., in Toxicology: Mechanisms and Analytical Methods (Ed. C.P. Stewart and A. Stotman) Vol. II, (1961).
6. Friedman, M.A., Greene, E.J., Csillag, R.G., and Epstein, S.S., Toxicol. and Appl. Pharmacol., 21:419 (1972).
7. Cohen, B.S., and Estabrook, R.W., Arch. Biochem. Biophys., 143:37 (1971).
8. Wattenberg, L.W., Leong, J.L., and Stroend, P.J.. Cancer Res., 22:1120 (1962).
9. Montesano, R., and Magee, P.N., Nature 228:173 (1970).
10. Wisbel, F.J., Lentz, Diamond, L., and Gelboin, H.V., Arch. Biochem. Biophys., 144:78 (1971).
11. Legator, M.S., and Malling, H.V., in Chemical Mutagens Ed. A. Hollender, Vol 2, 569 (1971).
12. Couch, D.B., McClanahan, H., and Friedman, M.A., Fed. Proc. 32:833, 1973.
13. Epstein, S.S., and Shafner, H., Nature 219:385 (1968).
14. DiPaolo, J.A., Nelson, R.L., and Donovan, P.J., Cancer Res., 31:1118 (1971).
15. DiPaolo, J.A., Nelson, R.L., Donovan, P.J., and Evans, C.H., Arch. Path. 95:380 (1973).
16. Flaks, A., Eur. J. Cancer, 6:259 (1970).
17. Toth, B., Magee, P. N., and Shubic, P., Cancer Res. 24:1712 (1964).
18. Lorenz, E., J. Natl. Canc. Inst. 1:17 (1940).

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Facilities Available

Laboratory facilities sufficient for the staff described here are available in McGuire Hall. The Pharmacology Department has sufficient animal quarters to allow a complete room for the sole use of mice in the carcinogenicity and mutagenicity studies described here. Another room will be used solely to house the inhalation exposure chambers. Available instruments include a Varian UV-visible double beam spectrophotometer, Packard Scintillation Counter, International Model J centrifuge, L-3 ultracentrifuge, Beckman gradient former, Isco gradient scanner, Labconco glassware washer, and others solely responsible to Dr. Friedman. Additionally, the Department of Pharmacology has five gas chromatographs, another ultracentrifuge, a Packard Oxidizer, two other scintillation counters, several fluorometers, I-R and N.M.R. spectrophotometers, mass spectrometer, CO_2 incubators, autoclave, tissue culture hoods, inverted scope, and a room solely devoted to tissue culture.

All carcinogenic material indlucing DMN and 3-MC will be prepared in a hazardous substance room. This room is equipped with a glove box and filtered exhaust fan. All personnel on this project will go through a training program to learn technique in handling these hazardous substances. This training program will be conducted by the principal investigator and Dr. Joseph F. Borzelleca who was Chairman of Standards Committee on Carcinogens for the Occupational Safety and Health Administration for the Department of Labor.

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11. Additional facilities required:

Dr. Friedman is director of the carcinogenesis program area of MCV/VCU Comprehensive Cancer Research Center (CCRC). Many of the individuals discussed here are part of this Program Area. The facilities and complete support of the CCRC will be available for this project.

Tissue and organ culture will be performed by Mrs. J. Munson. She currently maintains our tumor cell lines and determines direct cytotoxicity either with drugs or with lymphocytes and/or macrophages. She also has experience in cell transformation in vitro in our studies using Friend leukemia virus. Dr. Cribbs will consult on cell transformation data and mutagenicity experiments. Mr. J. G. Williams, Director of the Richmond Air Pollution Control Center, will collaborate in monitoring the NO_2 exposure levels. Similarly, he will perform relevant environmental surveillance of ambient NO_2 levels.

Histopathology will be performed with the collaboration of several pathologists. Everyday observation of the animals will be performed by Dr. Sawyer. Dr. Chesney, a veterinary pathologist, will be joining our staff soon. Dr. Richard Elzay and Dr. Frank Rea have aided in our histopathology in the past and will consult on this project.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

10. Continued

Studies involving mice will conform with the GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS prepared by the Institute of Laboratory Animal Resources, National Research Council (DHEN publication no. (NIH) 73-23) and with the federal laws and regulations. The quality of animal care will be scrutinized by the MCV/VCU Animal Care Committee and Dr. Chesney.

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Marvin A. Friedman	30	
Albert E. Munson	30	
John L. Egle, Jr.	30	
Richard P. Elzay	20	
Danny R. Sawyer	100	
Charles F. Chesney	30	
Technical		
Judith A. Munson	50	4402
Marilyn Green	50	4402
Kathleen Watt	50	4402
Walter Bullock	100	5943

Sub-Total for A: 19149

B. Consumable supplies (by major categories)

Chemicals	2500
Glassware	2000
Animals	3000
Animal Care	1000
Semisynthetic Diets	2000

Sub-Total for B: 10500

C. Other expenses (itemize)

Travel	500
Histopathology	1500
Chart Drawings and Photography	200
Page Charges & Reprints	300
Telephone	100
Postage	50
Laundry	50

Sub-Total for C: 2700 2200

Running Total of A + B + C: 32349

D. Permanent equipment (itemize)

Exposure Chambers	2000

Sub-Total for D: 34349

E. Indirect costs (15% of A+B+C): 5152 4,852

Total request: 39501 39,201

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	20298	13000	6000	2000	6195	47493
Year 3	21516	13000	6000	2000	6377	48893

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Nitrosamine: An Environmental Hazard	NIEHS: ES00713	98,168	Jan 1, 1972-Dec 31,1974

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Interactive Effects of of Piperonyl Butoxide	NIEHS: ES00925	89,990	March 1,1974-Feb 28,1977

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to
[illegible]cer Lossing, Controller

Mailing address for checks
1201 East Broad Street
Richmond, Virginia 23298

Principal investigator
Typed Name Marvin A. Friedman, Ph.D.
Signature Marvin A Friedman Date 1-31-74
Telephone 804 (Area Code) 770 (Number) 4670 (Extension)

Responsible officer of institution
Typed Name L. A. Woods, M. D., Ph.D.
Title Vice President for Health Sciences
Signature L A Woods Date 1-31-74
Telephone 804 (Area Code) 770 (Number) 4001 (Extension)

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